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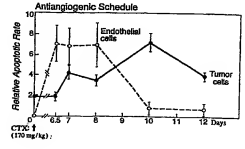

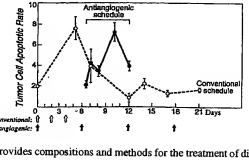

(57) Abstract: The present invention provides compositions and methods for the treatment of diseases associated with the abnormal growth of cells, and more specifically cancer. The compositions and methods of the present invention provide a decreased dosage of chemotherapeutic and antiangionic agents. In particular, the present invention provides methods of administering low doses of endostatin protein. Also included are methods of antiangiogenic scheduling of one or more therapeutic agents.

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METHODS FOR ADMINISTRATION OF THERAPEUTIC AGENTS ON AN ANTIANGIOGENIC SCHEDULE

Cross Reference to Related Applications

This application claims priority to provisional United States Application Serial No. 60/194,150, filed April 3, 2000 and United States Application Serial No. 09/439,901, filed on November 12, 1999 which are hereby incorporated by reference.

Field of the Invention

The present invention relates to the field of treating diseases associated with the abnormal growth of cells. In particular, the present invention relates to administration of low doses of therapeutic agents, such as endostatin, on an antiangiogenic schedule.

Background of the Invention

As used herein, the term "angiogenesis" means the generation of new blood vessels into a tissue or organ. Under normal physiological conditions, humans or animals undergo angiogenesis only in very specific restricted situations. For example, angiogenesis is normally observed in wound healing, fetal and embryonal development and formation of the corpus luteum, endometrium and placenta. However, angiogenesis also occurs under abnormal or undesired conditions such as during tumor development, growth and metastasis. This type of angiogenesis may also be referred to as uncontrolled angiogenesis.

Both controlled and uncontrolled angiogenesis are thought to proceed in a similar manner. Endothelial cells and pericytes surrounded by a basement membrane form capillary blood vessels. Angiogenesis begins with the erosion of the basement membrane by enzymes released by endothelial cells and leukocytes. The endothelial cells, which line the lumen of blood vessels, then protrude through the basement membrane. Angiogenic stimulants induce the endothelial cells to migrate through the eroded basement membrane. The migrating cells form a "sprout" off the parent blood vessel, where the endothelial cells undergo mitosis and proliferate. The endothelial sprouts merge with each other to form capillary loops, creating the new blood vessel.

Persistent, unregulated angiogenesis occurs in a multiplicity of disease states, tumor metastasis and abnormal growth by endothelial cells. The diverse pathological disease states in which unregulated angiogenesis is present have been grouped together as angiogenic dependent or angiogenic associated diseases. The hypothesis that tumor growth is angiogenesis-dependent was first proposed in 1971 by M. Judah Folkman. (Folkman J., "Tumor angiogenesis: Therapeutic implications" *N. Engl. Jour. Med.* 285:1182-1186 (1971)). In its simplest terms the hypothesis states: "Once tumor 'take' has occurred, every increase in tumor cell population must be preceded by an increase in new capillaries converging on the tumor." Tumor 'take' is currently understood to indicate a pre-vascular phase of tumor growth in which a population of tumor cells occupying a few cubic millimeters volume and not exceeding a few million cells, can survive on existing host microvessels. Expansion of tumor volume beyond this phase requires the induction of new capillary blood vessels.

Angiogenesis not only provides the increased nutrients and pathways for the removal of waste needed for the expansion of the tumor, but it also facilitates tumor metastasis by providing a route for tumor cells to leave the primary site and

enter the bloodstream (Zetter, 1998). In particular, angiogenesis increases the entry of tumor cells into the bloodstream by providing an increased density of immature, highly permeable blood vessels that have thinner basement membranes and fewer intracellular junction complexes than normal mature vessels (Zetter, 1998).

Since anti-cancer cytotoxic chemotherapy was first introduced fifty years ago, the repertoire of drugs directed against tumor cells has greatly increased (Farber *et al.*, 1948). Pre-clinical studies of experimental cancer therapies in mice conducted in the 1960s determined that one of several chemotherapy schedules tested, the maximum tolerated dose, yielded a higher percentage cure rate (Skipper *et al.*, 1964). This schedule, which consisted of the highest survivable (minimum lethal) dose, was chosen for the conventional administration of chemotherapy to cancer patients. However, such high, up-front doses require an extended treatment-free period to permit recovery of normal host cells, e.g., rapidly growing hematopoietic progenitors, that provides a window for reoccurrence and/or continued growth of the tumor. This extended treatment-free period also creates a high risk of selection for drug-resistance tumors due to the genetic instability and high mutation rate of neoplastic cells (Donehower *et al.*, 1992).

Despite the focus on the isolation of antiangiogenic agents, many antiangiogenic agents fail to completely regress or prevent the reoccurrence of a tumor. For example, the angiogenesis inhibitor TNP-470 has been reported to slow the growth, but not to regress drug-sensitive Lewis lung carcinoma (Brem *et al.*, 1993). Even combination therapy making use of antiangiogenic and chemotherapeutic agents such as TNP-470, cyclophosphamide, and minocycline have only yielded a 40-50% cure rate (Teicher *et al.*, 1994). Notably, what has not been described in the prior art is an optimal method of administering chemotherapeutic and antiangiogenic agents that lowers the

dosage and/or increases the effectiveness of these chemotherapeutic and antiangiogenic agents. In particular, the prior art has not described a method of therapeutic scheduling that focuses on the role of angiogenesis in diseases and conditions associated with the abnormal growth of cells. The prior art also has not described low dose administration of antiangiogenic agents such as endostatin.

Therefore, what is needed in the art are compositions and methods for the treatment of diseases associated with the abnormal growth of cells, such as cancer, that increase the efficiency of the chemotherapeutic and/or antiangiogenic agents used for treatment. More particularly, compositions and methods are needed for the treatment of diseases associated with the abnormal growth of cells that reduce the dose of chemotherapeutic or antiangiogenic agent required for treatment. Still further, compositions and methods for the treatment of cancer are needed that reduce the occurrence of drug resistant tumors.

Summary of the Invention

The present invention addresses the continuing need for improved methods of treatment of diseases associated with the abnormal growth of cells, and accordingly relates to compositions and methods for the treatment of diseases associated with the abnormal growth of cells. In particular, the present invention provides methods of administering low doses of therapeutic agents. The therapeutic agents as described herein are chemotherapeutic agents and antiangiogenic agents. In one aspect of the invention, the antiangiogenic agent is endostatin protein.

The methods of the present invention include antiangiogenic scheduling of one or more chemotherapeutic agents, one or more antiangiogenic agents, and a combination of one or more chemotherapeutic agents and one or more antiangiogenic agents. The present invention additionally

provides the chemotherapeutic and antiangiogenic agents used in the methods described herein.

The compositions and methods of the present invention also address the continuing need for methods of treatment that reduce the occurrence of drug resistant tumors. As defined herein, the term "antiangiogenic scheduling" refers to the administration of one or more therapeutic agents in such a manner as to reduce vascularization or re-vascularization of a tumor during a treatment schedule. In one aspect of the present invention, the antiangiogenic schedule provides a sustained apoptosis of the vascular endothelial cells in the tumor bed. A preferred antiangiogenic schedule comprises the administration of a therapeutic agent every four to eight days, and a more preferred antiangiogenic schedule comprises the administration of a therapeutic agent every six days.

In one embodiment of the present invention, a single chemotherapeutic agent such as cyclophosphamide is administered on an antiangiogenic schedule. In another embodiment of the present invention, a single antiangiogenic agent, such as endostatin protein, angiostatin protein or TNP-470 is administered on an antiangiogenic schedule. In yet another aspect of the present invention, a combination of chemotherapeutic and antiangiogenic agents are administered on an antiangiogenic schedule.

Accordingly, it is an object of the present invention to provide compositions and methods for the treatment of diseases associated with the abnormal growth of cells that are improved over the prior art.

It is another object of the present invention to provide compositions and methods for the treatment of cancer that are improved over the prior art.

It is another object of the present invention to provide compositions and methods of treatment that reduce the occurrence of drug resistant tumors.

It is another object of the present invention to provide compositions and methods for antiangiogenic scheduling.

It is another object of the present invention to provide compositions and methods for the treatment of diseases associated with the abnormal growth of cells that provide a more sustained apoptosis of the vascular endothelial cells in the tumor bed.

It is yet another object of the present invention to provide methods for antiangiogenic scheduling of chemotherapeutic agents.

It is another object of the present invention to provide methods for antiangiogenic scheduling of antiangiogenic agents.

It is an object of the present invention to provide methods for antiangiogenic scheduling of a combination of chemotherapeutic and antiangiogenic agents.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

Brief Description of the Drawings

Figure 1(a-b). Figure 1 is a graph showing antiangiogenic versus conventional scheduling of cyclophosphamide for drug-resistant Lewis lung carcinoma. Open triangles - control saline, open circles - conventional schedule (150 mg/kg every other day for 3 doses (white arrows, total 450 mg/kg) every 21 days), closed circles - antiangiogenic schedule (170 mg/kg every 6 days, CTX, thin black arrows), closed squares - antiangiogenic schedule of cyclophosphamide and TNP-470 (170 mg/kg cyclophosphamide and 12.5 mg/kg TNP-470 administered on the same day of the 6 day cycle for 7 cycles, CTX + TNP, thick black arrows).

Figure 2. Figure 2 is a table showing the *in vitro* anti-endothelial effects of activated cyclophosphamide on bovine capillary endothelial cell migration, survival and proliferation,

apoptosis and cell cycle distribution. 4-hydroperoxycyclophosphamide, which spontaneously converts to 4-HC in aqueous solution, was added at the indicated concentrations (Conc.). Values shown are the mean ± the standard error of the mean. Relative cell number refers to remaining, adherent endothelial cells (>600 fi) from an initial plating of 12,500 cells. Apoptosis was determined as a percentage of 10,000 intact (gated) cells by fluorescent flow cytometry. Cell cycle analysis was determined similarly using the ModFit LT software.

Figure 3(a-b). Figure 3(a) is a graph showing endothelial cell apoptosis precedes tumor cell apoptosis in cyclophosphamide-resistant Lewis lung carcinoma. Apoptotic rates on the antiangiogenic schedule of cyclophosphamide for endothelial cells are denoted as open circles, dashed line and apoptotic rates on the antiangiogenic schedule of cyclophosphamide for tumor cells are denoted as closed circles, solid line. Day 0 reflects analysis of 2 control tumors harvested at 100-200 mm^3. Day 0 reflects analysis of two control tumors harvested at 100-200 mm^3.

Figure 3(b) is a graph showing tumor cell apoptotic rates in drug-resistant Lewis lung carcinoma treated with the conventional schedule (open circles dotted line, white arrows) and antiangiogenic schedule (closed circles, solid line, black arrows). Day 0 reflects analysis of 2 control tumors harvested at 100-200 mm^3.

Figure 4. Figure 4 is a graph showing growth of drug-resistant Lewis lung carcinoma in p53 +/+ (dashed line) versus p53
-/- (solid line) C57B16/J mice treated on the antiangiogenic schedule of cyclophosphamide (170 mg/kg every six days, black arrows). Mice were treated as described in Figure 1 except that therapy was discontinued after 3 cycles in the p53 -/- mice to prevent the development of cardiotoxicity.

Detailed Description

The present invention provides compositions and methods for the treatment of diseases associated with the abnormal growth of cells, and more specifically cancer. The compositions and methods of the present invention decrease the dose administration of antiangiogenic and chemotherapeutic agents. The present invention also addresses the continuing need for methods of treatment that reduce the occurrence of drug resistant tumors. In particular, the present invention provides methods of antiangiogenic scheduling of a therapeutic agent.

In one aspect of the present invention, antiangiogenic agents are administered to an individual in need of treatment at a low dose. As defined herein, the term "antiangiogenic agent" refers to a composition that is capable of reducing the formation or growth of blood vessels. Examples of antiangiogenic agents include, but are not limited to, endostatin protein, angiostatin protein, TNP-470, angiozyme, anti-VEGF, apra (CT2584), bay 12-9566, benefin, BMS275291, bryostatin-1 (SC339555), CAI, carboxyamido-imidazole, CM101, combretastatin, dexrazoxane (ICRF187), DMXAA, EMD 121974, flavopiridol, GTE, IM862, interferon-alpha, interlukin-12, inhibitors of matrix metalloproteinases such as marimastat, metaret, metastat, MMI-270, neovastat (AE-941), octreotide (somatostatin), paclitaxel (taxol), penicllamine, photopoint, PI-88, prinomastat (AG-3340), purlytin, PTK787, squalamine, suradista (FCE26644), SU101, SU5416, SU6668, tamoxifen (nolvadex), tetrathiomolybdate, thalidomide, vitaxin and xeloda (capecitabine), cycloogenase, platelet factor 4 (PF-4), an N-terminally truncated proteolytically cleaved PF-4 fragment as described in Gupta, et al., 1995, a 16 kDa N-terminal fragment of human prolactin as described in Clapp et al., 1993, smaller protein fragments of fibronectin, murine epidermal growth factor, and thrombospondin as described in Homandberg et al., 1985, Nelson et al., 1995, and Tolsma et al., 1993.

In one embodiment, a low dose of endostatin protein is administered to an individual. As used herein, the term "endostatin protein" refers to a C-terminal region fragment of a collagen molecule that has antiangiogenic activity *in vivo*. Examples of collagen molecules include, but are not limited to, collagen XVIII, collagen XV and collagen IV. Examples of endostatin proteins may also be found in U.S. Patent No. 5,854,205 which is hereby incorporated by reference. The present invention provides methods of administering low doses of endostatin protein for the treatment of angiogenesis-related diseases and conditions. In one embodiment, endostatin protein is administered at a dose of less than approximately 0.3 mg/kg/day, more preferably, between approximately 0.01 to 0.1 mg/kg/day and most preferably, between approximately 0.03 to 0.08 mg/kg/day. In another embodiment, endostatin protein is administered to an individual wherein tumor development has progressed to a late stage at a dose of between approximately 1 to 20 mg/kg/day, more preferably between approximately 2 to 10 mg/kg/day, and most preferably between approximately 4 to 6 mg/kg/day.

Additionally, as used herein, the term "angiostatin protein" refers to a kringle region fragment of a plasminogen molecule that has antiangiogenic activity *in vivo*. Examples of angiostatin proteins may be found in U.S. Patent No. 5,837,682 and U.S. Patent No. 5,854,221 which are hereby incorporated by reference. Plasminogen contains five kringle region fragments, denoted kringles 1-5, as well as inter-kringle regions. It is to be understood that the term "angiostatin protein" refers to any single kringle region, any combination of kringle regions, or any kringle regions in addition to any inter-kringle regions that retain antiangiogenic activity *in vivo*. In a preferred embodiment, angiostatin protein is approximately kringle regions 1-3, kringle regions 1-3.5, kringle regions 1-4 or kringle regions 1-4.5 of human plasminogen. In another preferred embodiment,

angiostatin protein comprises kringle regions 1-4.5 of human plasminogen.

The terms "endostatin protein" and "angiostatin protein" also include shortened proteins wherein one or more amino acid is removed from either or both ends of an endostatin protein or an angiostatin protein, respectively, or from an internal region of either protein, yet the proteins retain angiogenesis inhibiting activity *in vivo*. The terms "endostatin protein" and "angiostatin protein" also include lengthened proteins or peptides wherein one or more amino acids is added to either or both ends of an endostatin protein or an angiostatin protein, respectively, or to an internal location, yet the proteins retain angiogenesis inhibiting activity *in vivo*. Labeling endostatin protein and angiostatin protein with other radioisotopes or chemicals such as ricin may also be useful in providing a molecular tool for destroying the target cells containing endostatin protein or angiostatin protein receptors.

Also included within the terms "angiostatin protein" and "endostatin protein" are angiostatin protein and endostatin protein derivatives. An angiostatin protein derivative includes a protein having the amino acid sequence of a kringle region fragment of a plasminogen that has antiangiogenic activity. An angiostatin protein also includes a peptide having a sequence corresponding to an antiangiogenic angiostatin fragment of a kringle region fragment of a plasminogen. An "antiangiogenic angiostatin fragment" is defined to be a peptide whose amino acid sequence corresponds to a subsequence of a kringle region fragment of a plasminogen, referred to as an "antiangiogenic angiostatin subsequence".

An endostatin protein derivative includes a protein having the amino acid sequence of a C-terminal region fragment of a collagen molecule that has antiangiogenic activity. An endostatin protein also includes a peptide having a sequence corresponding to an antiangiogenic endostatin fragment of a C-terminal region fragment of a collagen molecule. An

"antiangiogenic endostatin fragment" is defined to be a peptide whose amino acid sequence corresponds to a subsequence of a C-terminal region fragment of a collagen molecule, referred to as an "antiangiogenic endostatin subsequence". A "subsequence" is a sequence of contiguous amino acids found within a larger sequence. A subsequence is generally composed of approximately at least 70%, more preferably 80%, and most preferably 90% of the larger sequence.

Angiostatin and endostatin protein derivatives also include a protein or peptide having a modified sequence in which one or more amino acids in the original sequence or subsequence have been substituted with a naturally occurring amino acid residue or amino acid residue analog (also referred to as modified amino acid). Such substitutions may modify the bioactivity of angiostatin protein and angiostatin protein, such as by increasing or decreasing the angiogenesis inhibiting activity, and produce biological or pharmacological agonists or antagonists. Suitable angiostatin and endostatin derivatives have modified sequences which are substantially homologous to the amino acid sequence of an angiostatin and endostatin protein, respectively, or to an antiangiogenic subsequence of an angiostatin and endostatin protein, respectively.

An "amino acid residue" is a moiety found within a protein or peptide and is represented by -NH-CHR-CO-, wherein R is the side chain of a naturally occurring amino acid. When referring to a moiety found within a peptide, the terms "amino acid residue" and "amino acid" are used interchangeably. An "amino acid residue analog" includes D or L configurations having the following formula: -NH-CHR-CO-, wherein R is an aliphatic group, a substituted aliphatic aromatic group, a benzyl group, a substituted benzyl group, an aromatic group or a substituted aromatic group and wherein R does not correspond to the side chain of a naturally occurring amino acid.

Suitable substitutions for amino acid residues in the sequence of the angiostatin and endostatin proteins described

herein include conservative substitutions that result in angiogenic angiostatin and endostatin protein derivatives. A conservative substitution is a substitution in which the substituting amino acid (naturally occurring or modified) is structurally related to the amino acid being substituted. "Structurally related" amino acids are approximately the same size and have the same or similar functional groups in the side chains.

Provided below are groups of naturally occurring and modified amino acids in which each amino acid in a group has similar electronic and steric properties. Thus, a conservative substitution can be made by substituting an amino acid with another amino acid from the same group. It is to be understood that these groups are non-limiting and that additional modified amino acids could be included in each group.

Group I includes leucine, isoleucine, valine, methionine and modified amino acids having the following side chains: ethyl, *n*-propyl *n*-butyl. Preferably, Group I includes leucine, isoleucine, valine and methionine.

Group II includes glycine, alanine, valine and a modified amino acid having an ethyl side chain. Preferably, Group II includes glycine and alanine.

Group III includes phenylalanine, phenylglycine, tyrosine, tryptophan, cyclohexylmethyl, and modified amino residues having substituted benzyl or phenyl side chains. Preferred substituents include one or more of the following: halogen, methyl, ethyl, nitro, $-NH_2$, methoxy, ethoxy and –CN. Preferably, Group III includes phenylalanine, tyrosine and tryptophan.

Group IV includes glutamic acid, aspartic acid, a substituted or unsubstituted aliphatic, aromatic or benzylic ester of glutamic or aspartic acid (e.g., methyl, ethyl, *n*-propyl *iso*-propyl, cyclohexyl, benzyl or substituted benzyl), glutamine, asparagine, -CO-NH-alkylated glutamine or asparagine (e.g., methyl, ethyl, *n*-propyl and *iso*-propyl) and modified amino acids having the side chain $-(CH_2)_3$-COOH, an ester thereof (substituted

or unsubstituted aliphatic, aromatic or benzylic ester), an amide thereof and a substituted or unsubstituted N-alkylated amide thereof. Preferably, Group IV includes glutamic acid, aspartic acid, methyl aspartate, ethyl aspartate, benzyl aspartate and methyl glutamate, ethyl glutamate and benzyl glutamate, glutamine and asparagine.

Group V includes histidine, lysine, ornithine, arginine, N-nitroarginine, β-cycloarginine, γ-hydroxyarginine, N-amidinocitruline and 2-amino-4-guanidinobutanoic acid, homologs of lysine, homologs of arginine and homologs of ornithine. Preferably, Group V includes histidine, lysine, arginine and ornithine. A homolog of an amino acid includes from 1 to about 3 additional or subtracted methylene units in the side chain.

Group VI includes serine, threonine, cysteine and modified amino acids having C1-C5 straight or branched alkyl side chains substituted with –OH or –SH, for example, -CH_2CH_2OH, -$CH_2CH_2CH_2OH$ or –$CH_2CH_2OHCH_3$. Preferably, Group VI includes serine, cysteine or threonine.

In another aspect of the present invention, suitable substitutions for amino acid residues in the amino acid sequences described herein include "severe substitutions" that result in angiostatin and endostatin protein derivatives that are antiangiogenic. Severe substitutions that result in antiangiogenic angiostatin and endostatin protein derivatives are much more likely to be possible in positions that are not highly conserved than at positions that are highly conserved. In the present invention, severe substitutions are much more likely to be possible in the inter-kringle regions of angiostatin. A "severe substitution" is a substitution in which the substituting amino acid (naturally occurring or modified) has significantly different size and/or electronic properties compared with the amino acid being substituted. For example, the side chain of the substituting amino acid can be significantly larger (or smaller) than the side chain of the amino acid being substituted and/or can have functional

groups with significantly different electronic properties than the amino acid being substituted.

Examples of severe substitutions of this type include the substitution of phenylalanine or cyclohexylmethyl glycine for alanine, isoleucine for glycine, a D amino acid for the corresponding L amino acid or –NH-CH[(-CH_2)$_5$-COOH]-CO- for aspartic acid. Alternatively, a functional group may be added to the side chain, deleted from the side chain or exchanged with another functional group. Examples of severe substitutions of this type include adding an amine or hydroxyl, carboxylic acid to the aliphatic side chain of valine, leucine or isoleucine, exchanging the carboxylic acid in the side chain of aspartic acid or glutamic acid with an amine or deleting the amine group in the side chain of lysine or ornithine. In yet another alternative, the side chain of the substituting amino acid can have significantly different steric and electronic properties that the functional group of the amino acid being substituted. Examples of such modifications include tryptophan for glycine, lysine for aspartic acid and –(CH_2)$_4$COOH for the side chain of serine. These examples are not meant to be limiting.

"Substantial homology" exists between two amino acid sequences when a sufficient number of amino acid residues at corresponding positions of each amino acid sequence are either identical or structurally related such that a protein or peptide having the first amino acid sequence and a protein or peptide having the second amino acid sequence exhibit similar biological activities. Generally, there is substantial sequence homology among the amino acid sequences when at least 30%, more preferably at least 40%, and most preferably at least 50%, of the amino acids in the first amino acid sequence are identical to or structurally related to the second amino acid sequence. Homology is often measured using sequence analysis software, e.g., BLASTIN or BLASTP. The default parameters for comparing the two sequences (e.g., "Blast"-ing two sequences against each other) by BLASTIN (for nucleotide sequences) are reward for

match =1, penalty for mismatch = -2, open gap = 5, and extension gap = 2. When using BLASTP for protein sequences, the default parameters are reward for match = 0, penalty for mismatch = 0, open gap = 11, and extension gap = 1.

As used herein, the terms "endostatin protein" and "angiostatin protein" also refer to fusion proteins containing endostatin protein and/or angiostatin protein. In one aspect of the present invention, angiostatin protein and endostatin protein are recombinantly fused together into a single protein molecule. Endostatin protein and angiostatin protein may also be recombinantly fused to a Fc portion of an antibody.

In another aspect of the invention, a therapeutic agent is administered on an antiangiogenic schedule. As defined herein, the term "antiangiogenic schedule" refers to the administration of one or more therapeutic agents in such a manner as to reduce vascularization or re-vascularization of a tumor during a treatment schedule. It is to be understood that as defined herein, the term "antiangiogenic scheduling" includes, but is not limited to, modification of the timing of the administration of a therapeutic agent in order to reduce vascularization or re-vascularization of a tumor during a treatment schedule, modification of the amount of the administration of a therapeutic agent in order to reduce vascularization or re-vascularization of a tumor during a treatment schedule, and modification of the formulation of a therapeutic agent in order to reduce vascularization or re-vascularization of a tumor during a treatment schedule. In a preferred aspect, the antiangiogenic schedule provides a relatively sustained apoptosis of the vascular endothelial cells in the tumor bed.

In one aspect of the present invention, the antiangiogenic schedule comprises the administration of a therapeutic agent every four to eight days, and more preferably every six days. In a further preferred embodiment, the antiangiogenic schedule comprises the administration of cyclophosphamide every six days. In an alternate embodiment,

doxil is administered on an antiangiogenic schedule of every six days. In another aspect of the present invention, the antiangiogenic schedule comprises the administration of a therapeutic agent by continuous infusion. In a further preferred embodiment, the antiangiogenic schedule comprises the administration of 5-fluorouracil or 6-mercaptopurine by continuous infusion. In yet another aspect of the present invention, the antiangiogenic schedule comprises the administration of a therapeutic agent that is encapsulated into liposomes or conjugated to vascular integrin-binding peptides. In a further preferred embodiment, the antiangiogenic schedule comprises the administration of a liposomal encapsulation of doxorubicin (doxil) or doxorubicin conjugated to vascular integrin-binding peptides.

As defined herein, the term "therapeutic agent" refers to a pharmaceutical composition, a nutraceutical composition or radiation. Examples of pharmaceutical compositions include, but are not limited to, chemotherapeutic agents and antiangiogenic agents. The term "nutraceutical composition" refers to a natural, or non-synthetic, composition that provides health benefits to an individual to whom the composition is administered. The methods include antiangiogenic scheduling of one or more chemotherapeutic agents, one or more antiangiogenic agents, and a combination of one or more chemotherapeutic agents and one or more antiangiogenic agents. The present invention additionally provides the chemotherapeutic and antiangiogenic agents used in the methods described herein.

In a further aspect of the present invention, one or more chemotherapeutic agents are administered on an antiangiogenic schedule to an individual in need of such a treatment. As used herein, the term "chemotherapeutic agent" refers to a chemical composition used for the treatment or control of a disease in an individual. Examples of chemotherapeutic agents include, but are not limited to, 6-mercaptopurine, dacarbazine, l-asparaginase, procarbazine, raloxifen, tamoxifen,

antimetabolites such as cytarabine, 5-fluorocil, gemcitabine, cladribine, fludarabine, pentostatin, and hyroxyurea, plant alkaloids such as docetaxel, paclitaxel, vinblastine, vincristine, and vinorelbine, topoisomerase inhibitors such as daunorubicin, doxorubicin, idarubicin, etoposide, teniposide, dactinomycin and mitoxantrone, alkylating agents such as busulfan, chlorambucil, cyclophosphamide, ifosfamide, mecholorethamine, melphalan, thiotepa, carmustine, lomustine, carboplatin and cisplatin, and anti-tumor antibiotics such as bleomycin and mitomycin C.

In a preferred aspect of the invention, cyclophosphamide is administered on an antiangiogenic schedule to an individual in need of such a treatment. In a further preferred embodiment, cyclophosphamide is administered to an individual in need of such a treatment on an antiangiogenic schedule, wherein the antiangiogenic schedule comprises administration every four to eight days, and more preferably every six days. As described in more detail in the Examples, the six day antiangiogenic schedule of cyclophosphamide: (1) increased endothelial cell apoptosis in the tumor bed; (2) demonstrated long-term suppression of the growth of cyclophosphamide-resistant Lewis lung carcinoma, a significant improvement over the conventional schedule; (3) eradicated drug-sensitive Lewis lung carcinoma by avoiding acquired drug-resistance, an outcome not possible with a conventional schedule; and (4) eradicated the majority of drug-resistant Lewis lung carcinomas when combined with another angiogenesis inhibitor, TNP-470.

In yet another aspect of the present invention, a combination of chemotherapeutic and antiangiogenic agents are administered on an antiangiogenic schedule. In a preferred aspect, cyclophosphamide and TNP-470 are administered on an antiangiogenic schedule to an individual in need of such a treatment. In a further preferred embodiment, the cyclophosphamide and TNP-470 are administered on the same day of a six-day treatment cycle.

The compositions and methods described above are useful for the treatment of diseases and conditions related to the abnormal growth or proliferation of cell. In particular, the compositions and methods of the present invention are useful for the treatment of metastatic and angiogenesis-dependent cancers and other angiogenesis-related diseases. One example of metastatic disease is metastatic cancer. Angiogenesis-related diseases include, but are not limited to, angiogenesis-dependent cancer, including, for example, solid tumors, blood born tumors such as leukemias, and tumor metastases; benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; rheumatoid arthritis; psoriasis; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis; Osler-Webber Syndrome; myocardial angiogenesis; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; and wound granulation. The HA binding proteins of the present invention are useful in the treatment of disease of excessive or abnormal stimulation of endothelial cells. These diseases include, but are not limited to, intestinal adhesions, atherosclerosis, scleroderma, and hypertrophic scars, i.e., keloids. They are also useful in the treatment of diseases that have angiogenesis as a pathologic consequence such as cat scratch disease (*Rochele minalia quintosa*) and ulcers (*Helobacter pylori*).

The antiangiogenic agents described above can be provided as isolated and substantially purified proteins and protein fragments. Both the antiangiogenic and chemotherapeutic agents described herein can be provided in pharmaceutically acceptable formulations using formulation methods known to those of ordinary skill in the art. The antiangiogenic and chemotherapeutic agents described above may be a solid, liquid or aerosol. Examples of solid therapeutic compositions include pills, creams, and implantable dosage units. The pills may be

administered orally and the therapeutic creams may be administered topically. The implantable dosage units may be administered locally, for example at a tumor site, or may be implanted for systemic release of the therapeutic angiogenesis-modulating composition, for example subcutaneously. Examples of liquid compositions include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Examples of aerosol formulations include inhaler formulations for administration to the lungs. In general however, the formulations may be administered by any route, including but not limited to, the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (e.g., intravenous, intraspinal, subcutaneous or intramuscular) route.

In addition, the antiangiogenic and chemotherapeutic agents may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor or implanted so that the antiangiogenic or chemotherapeutic agent is slowly released systemically. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.*, "Interstitial chemotherapy with drug polymer implants for the treatment of recurrent gliomas" *J. Neurosurg.* 74:441-446 (1991). Osmotic minipumps may also be used to provide controlled delivery of high concentrations of antiangiogenic and chemotherapeutic agents through cannulae to the site of interest, such as directly into a metastatic growth or into the vascular supply to that tumor.

The dosage of the antiangiogenic and chemotherapeutic agents of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. The present invention provides compositions and methods for administering a

low dose of chemotherapuetic and/or antiangiogenic agent. With regard to administration of endostatin protein, a "low dose" refers to a dosage between approximately 0.001 mg/kilogram per day to 0.3 mg/kilogram per day, more preferably 0.01 to 0.1 mg/kilogram per day, and most preferably 0.03 to 0.08 mg/kilogram per day.

Depending upon the half-life of the antiangiogenic or chemotherapeutic agent in the particular animal or human, it can be administered between several times per day to once a week. In one aspect of the invention, an antiangiogenic agent is administered to a human or animal on an antiangiogenic schedule, wherein the antiangiogenic schedule comprises administration every four to eight days, and more preferably every six days. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The therapeutic agent formulations may conveniently be presented in unit dosage form and may be prepared by conventional pharmaceutical techniques. Such techniques include the step of bringing into association the active ingredient and the pharmaceutical carrier(s) or excipient(s). Suitable pharmaceutical carriers and excipients are known to those skilled in the art, however, an example of a suitable pharmaceutical excipient is water. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose

containers, for example, sealed ampules or vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose, or an appropriate fraction thereof, of the administered ingredient. It should be understood that in addition to the ingredients, particularly mentioned above, the formulations of the present invention may include other agents conventional in the art having regard to the type of formulation in question. Optionally, cytotoxic agents may be incorporated or otherwise combined with the therapeutic agents described herein, or biologically functional protein fragments thereof, to provide dual therapy to the patient.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

Example 1

Eradication of drug-sensitive Lewis lung carcinoma by the antiangiogenic schedule of cyclophosphamide and prevention of acquired drug resistance

As shown in Figure 1(b), treatment of a drug-sensitive Lewis lung carcinoma comprising administration of cyclophosphamide on an antiangiogenic schedule eradicated tumors in 100% of mice. Figure 1(b) may be read as follows. Open triangles - control saline, open circles - conventional schedule (150 mg/kg every other day for 3 doses (white arrows,

total 450 mg/kg) every 21 days), closed circles - antiangiogenic schedule (170 mg/kg every 6 days, thin black arrows). The insert at upper right reveals magnified axes for the first 21 days of therapy, n=6/group. Therapy on the antiangiogenic schedule was discontinued after 7 cycles, 3 cycles beyond the point at which tumors were no longer visible. Three separate experiments produced similar results.

In contrast to similar initial tumor burdens of Lewis lung carcinoma that have acquired drug resistance on the conventional schedule, administration of cyclophosphamide on an antiangiogenic schedule resulted in long-term tumor-free survival. The eradication of Lewis lung carcinoma was interpreted as resulting from two actions of cyclophosphamide: (i) the direct cell-kill of drug-sensitive tumor cells and (ii) the direct cell-kill of endothelial cells leading to the apoptosis of both drug-sensitive and, more importantly, drug-resistant tumor cells.

Example 2

Determination of an optimum antiangiogenic dosing schedule for cyclophosphamide

In order to determine the optimum antiangiogenic dosing schedule for cyclophosphamide, a drug resistant tumor was selected for treatment. It was reasoned that by rendering this tumor completely drug-resistant, any dosing schedule that improved tumor control was likely to be the direct result of optimized antiangiogenic activity. The method of Teicher et al., 1990 was used to select a drug-resistant Lewis lung carcinoma by treatment of tumor-bearing mice with a supra-lethal dose of cyclophosphamide (500 mg/kg). After 24 hours, the tumor was passaged into syngeneic mice. A clinically resistant (cyclophosphamide-insensitive) tumor rapidly developed and selection was continued over eight cycles of passage and re-treatment.

Following the eighth cycle of selection, drug-resistant Lewis lung carcinoma was explanted into tissue culture

as described for the cyclophosphamide-resistant breast cancer cell line, EMT-6/CTX (Teicher et al., 1990). The drug-resistant Lewis lung carcinoma and the original, drug-sensitive Lewis Lung carcinoma were screened for mouse hepatitis virus and other pathogens, and stored in aliquots in liquid nitrogen. For tumor studies, cells were thawed and passaged once in C57B16/J mice (Jackson Labs, ME). When tumors reached 100-200 mm^3, mice harboring drug-resistant Lewis lung carcinomas received 170 mg/kg of cyclophosphamide subcutaneously every six days for two cycles and then the tumors were allowed to grow for transfer. Tumor inoculations, drug injections (including ondansetron and dexamethasone), and tumor measurements were carried out as described in Boehm et al., 1997 using 28-30 gram adult male C57B16/J mice (Jackson Labs, ME) free of viral pathogens. Mice in these experiments were fed a "Western Type" diet, 42% calories from fat (TD 88137, Harlan Teklad, Madison, WI) to ameliorate weight loss. These food pellets were placed on the floor of the cage.

Therapy was initiated 2-4 days following inoculation of the tumor, just as tumor volumes reached 100 mm^3. Cyclophosphamide was then administered daily or every 3, 4, 5, 6, 7, or 8 days to mice bearing the drug-resistant Lewis lung carcinoma. The dosing schedules were more widely spaced and more sustained than similar non-conventional schedules reported previously for Lewis lung carcinoma (Humphreys et al., 1970; Skipper et al., 1989), yet resulted in no more than a 5% weight loss over time. Cyclophosphamide administered at 170 mg/kg every six days proved more effective in controlling tumor growth than other cyclophosphamide schedules tested (including schedules with a higher dose-intensity, e.g. 135 mg/kg every 4 days, data not shown).

Figure 1(a) shows the comparison of the growth of drug-resistant Lewis lung carcinoma in mice treated with cyclophosphamide on a conventional schedule of the maximum tolerated dose (150 mg/kg every other day for three doses given

every 21 days = 450 mg/kg every 21 days) with tumor growth on an antiangiogenic schedule (170 mg/kg every 6 days). Figure 1(a) may be read as follows. Open triangles - control saline, open circles - conventional schedule (150 mg/kg every other day for 3 doses (white arrows, total 450 mg/kg) every 21 days), closed circles - antiangiogenic schedule (170 mg/kg every 6 days, CTX, thin black arrows), closed squares - antiangiogenic schedule of cyclophosphamide and TNP-470 (170 mg/kg cyclophosphamide and 12.5 mg/kg TNP-470 administered on the same day of the 6 day cycle for 7 cycles, CTX + TNP, thick black arrows). The insert at the upper right of Figure 1(a) has magnified axes for the first 21 days of therapy, n=6/group.

As shown in Figure 1(a), all control and conventional schedule mice died with large tumor burdens. Therapy was discontinued on the antiangiogenic schedule of cyclophosphamide alone after 2/6 mice died with pulmonary inflammation, accompanied by high peripheral leukocyte counts. No mouse on either schedule had visibly detectable pulmonary metastases at the time of death. On the conventional schedule, drug-resistant tumors escaped by day 13 and grew rapidly (Figure 1(a), insert). In addition, mice treated on the conventional schedule lost 21% of body weight, which was regained prior to the next treatment cycle. In contrast, on the antiangiogenic schedule there was no net tumor growth for 36 days and weight loss was less than 5%. Following the first seven cycles (36 days) of therapy on the antiangiogenic schedule, tumor growth occurred at a slow rate.

Example 3

Cyclophosphamide induces apoptosis of endothelial cells in vitro

The ability of cyclophosphamide to inhibit endothelial cell proliferation and induce apoptosis *in vitro* was determined as follows. For proliferation studies, 12,500 bovine adrenal capillary endothelial cells in DMEM and 10% bovine calf serum were plated onto gelatinized 24-well plates in quadruplicate. For apoptosis and cell cycle determinations, 2 x

10^6 cells were similarly split into TISO flasks. Sixteen hours later, the media was aspirated and replaced with DMEM and 5% bovine calf serum with or without 5 ng/ml bFGF (Scios, Mountain View, CA) as indicated.

Freshly reconstituted 4-hydroperoxycyclophosphamide (Omicron Biochemicals, San Antonio, TX), which spontaneously converts to 4-HC in aqueous solution, was added at the concentrations indicated in Figure 2. (Cyclophosphamide is a pro-drug that is modified *in vivo* by hepatic mixed function oxidases to create the active drug, 4-hydroxycyclophosphamide (4-HC)). Eighteen hours later, the cells were trypsinized and enumerated for proliferation as described in O'Reilly et al., 1994 or washed with phosphate buffered saline and incubated with annexin-fluorescein as per the ApoAlert Annexin V apoptosis detection kit (Clontech, Palo Alto, CA). Cells were then washed in cold phosphate buffered saline, fixed by drop-wise dispersion while vortexing into cold 80% ethanol, and incubated for 30 minutes on ice. Cells were again washed in cold phosphate buffered saline. Propidium iodide (Sigma, St. Louis, MO) and RNAase A (Boehringer-Mannheim, Indianapolis, IN) were added to give a concentration of 2.5 μg/ml and 50 μg/ml, respectively. Samples were incubated for 30 minutes at 37°C and analyzed by flow cytometry. For migration studies, bovine capillary endothelial cells were exposed to 4-HC as described above. Migration was performed as described in Moses et al., 1990 without adding additional 4-HC.

Thus, capillary endothelial cells were exposed for 16 hours to 4-HC *in vitro* at concentrations similar to those obtained *in vivo* (Kachel et al., 1994). The 4-HC drug induced a concentration-dependent cell cycle arrest and apoptosis of capillary endothelial cells (Figure 2). The majority of endothelial cells at high concentrations of 4-HC (10 μg/ml) arrested in G_1 and showed increased apoptosis. Lower concentrations (0.1 μg/ml 4-HO) were cytostatic and were associated with a prolongation of S phase. Importantly, when endothelial cell migration was

stimulated *in vivo* by bFGF, even these lower concentrations (0.1 μg/ml 4HC) caused a 45% decrease in migration (Figure 2) without affecting the protein levels of three integrins (data not shown).

Example 4

Cyclophosphamide inhibits angiogenesis in vivo

To demonstrate the effect of cyclophosphamide on growth factor-induced neovascularization *in vivo*, mouse corneas were implanted with bFGF pellets which stimulated corneal neovascularization over 5-6 days (O'Reilly et al., 1994). Treatment with a single dose of cyclophosphamide on a six day antiangiogenic schedule 24 hours after pellet implant (when limbal dilatation and vascular sprouts are just appearing) inhibited the area of new vessel growth by 66 ± 5% (data not shown). Treatment with the conventional schedule of cyclophosphamide, i.e., 3 doses of 150 mg/kg at 24, 72, and 120 hours, resulted in 73 ± 5% inhibition of new vessel growth (data not shown). It should be noted that comparison of levels of inhibition in corneal neovascularization is only valid until day six, because the bFGF stimulus fades.

While inhibition of corneal angiogenesis did not differ statistically between the two schedules, in a tumor-bearing mouse this anti-endothelial effect would occur 3.5 times on the six day antiangiogenic schedule, in contrast to one time on the 21-day conventional schedule. In mice bearing drug-resistant tumors, both the conventional and antiangiogenic schedules inhibited tumor growth during the first 13 days (see Figure 1(a), insert). Because the tumor cells are drug-resistant *in vivo*, these data suggest that tumor growth, which occurred following the first 13 days on the conventional schedule, was prevented on the antiangiogenic schedule by a more sustained inhibition of angiogenesis within the tumor bed by cyclophosphamide.

Example 5

Cyclophosphamide induces endothelial cell and tumor cell apoptosis in vivo

Additional evidence that cyclophosphamide controls drug-resistant Lewis lung carcinoma through endothelial cell inhibition is that cyclophosphamide induces *in vivo* apoptosis of endothelial cells prior to *in vivo* apoptosis of tumor cells. To determine whether cyclophosphamide induced endothelial cell apoptosis in the tumor bed, cell turnover was analyzed in drug-resistant tumors as follows.

Mice harboring drug-resistant Lewis lung carcinoma were injected with 0.5 ml of 10 mM BrdU (Boehringer-Mannheim, Indianapolis, IN) in phosphate buffered saline intraperitoneally one hour prior to being euthanized with Metofane (Mallinckrodt Veterinary, Mundelein, IL) followed by cervical subluxation. For mice on the conventional schedule, tumors on days 5, 7, 12, 14, 17, and 21 were analyzed for proliferation and apoptosis as previously described (Brem et al., 1993). For mice on the antiangiogenic schedule, tumors were analyzed on days 6.5, 7, 8, 10, and 12.

Tumors were resected and immediately fixed in cold buffered formalin, incubated overnight at 4°C, then changed into cold phosphate buffered saline, and paraffin embedded within 24 hours of excision. Tumor sections of 5 microns were deparaffinized. Antigen retrieval included 10mM EDTA pH 6.0 at 70°C for 5 minutes and cooling to room temperature for 20 minutes, followed by digestion with proteinase K (Boehringer Mannheim, Indianapolis, IN) 10 μg/ml in 0.1 M Tris pH 7.4 at 37°C for 20 minutes.

While BrdU incorporation in tumor cells was similar in control and cyclophosphamide-treated mice, endothelial cell and tumor cell apoptosis revealed marked differences between treatment groups (Figure 3(a)). As shown in Figure 3(b), untreated drug-resistant Lewis lung carcinoma cells showed a labeling index of 37% and a low apoptotic rate of 1.7% at small

tumor sizes. The conventional schedule generated one peak of tumor cell apoptosis that fell to background levels from day 12 through day 21 after the start of treatment. In contrast, one cycle of the antiangiogenic schedule generated an equivalent peak of tumor cell apoptosis over a six day period.

Double immunofluorescence (staining with von Willebrand factor antibody and TUNEL assay) was employed to discriminate endothelial cell apoptosis from tumor cell apoptosis. TUNEL assay was performed according to the fluorescein ApopTag kit (Oncor, Gaithersburg, MD). Slides were incubated with rabbit anti-human von Willebrand factor polyclonal antibody (Dako, Carpinteria, CA) 1:500 overnight at 4^{0}C. Biotinylated anti-rabbit secondary antibody was added, followed by Texas Red-avidin and anti-digoxigenin-fluorescein. Sections were co-stained with Hoechst 33258 (Sigma, St. Louis, MO). Slides were photographed using an Axiophot photomicroscope equipped with a Texas Red and fluorescein double filter (Zeiss, Oberkochen, Germany). The same field was then photographed using the Hoechst filter. Total endothelial cell apoptosis per microvessel count were tabulated per 157X field from projected 35-mm slides. Total tumor cell apoptosis was determined by counting tumor cell apoptotic nuclei per total Hoechst staining nuclei for each slide pair. Two independent observers obtained similar results.

On the antiangiogenic schedule, endothelial cell apoptosis preceded tumor cell apoptosis by 3.5 days, suggesting that the anti-endothelial effect of cyclophosphamide is primary and causative (data not shown). Because the half-life of cyclophosphamide in mice is less than 30 minutes (Mellett, 1971; Sladek, 1994) and the BrdU incorporation rate by tumor cells on the antiangiogenic schedule remained at 35% (similar to untreated controls), tumor cell apoptosis most likely resulted from endothelial cell suppression and not from delayed tumor penetration of activated cyclophosphamide.

Example 6

Drug-resistant tumor growth inhibition by cyclophosphamide is linked to endothelial cell p53

Further proof that endothelial cells are the main targets of cyclophosphamide in drug-resistant Lewis lung carcinoma was obtained in p53-null mice (Donehower et al., 1992). While the endothelial cells in these mice should be as susceptible to cyclophosphamide-mediated DNA damage (as p53 +/+ endothelium), they should be unable to fully repair this DNA damage or undergo apoptosis without the arrest of cell cycle mediated by p53. Thus, tumor growth in p53 +/+ mice would depend on p53-normal endothelial cells, while tumor growth in p53 -/- mice would depend on p53-null endothelial cells. Figure 4 compares the growth of drug-resistant Lewis lung carcinoma in p53 +/+ mice and p53 -/- mice treated on an antiangiogenic schedule wherein cyclophosphamide was administered every six days at 170 mg/kg. While tumor growth in p53 +/+ mice was completely suppressed for at least six 6-day cycles of cyclophosphamide, tumor growth in p53 -/- mice was not affected by the first dose of cyclophosphamide. Tumor cell proliferation, and tumor cell and endothelial cell apoptosis of these tumors in p53 -/- mice were all equivalent to untreated control tumors until day six. Within three hours following the second dose of cyclophosphamide on day six, greater than 90% of the mass of these now large, cyclophosphamide-resistant tumors in the p53 -/- mice underwent nearly synchronous apoptosis (data not shown). Following a third dose of cyclophosphamide, tumors in the p53-/- mice were eradicated and did not recur off therapy. The p53 -/- mice died of secondary tumors (expected in p53 -/-mice) within two months following therapy.

The above results demonstrated that cyclophosphamide is not directly cytotoxic to tumor cells per se, as evidenced by the initial rapid tumor growth in p53 -/- mice, and therefore, cyclophosphamide must be inhibiting tumor growth mainly through its cytotoxic effect on endothelial cells in

the tumor bed. In the p53 +/+ mice, cyclophosphamide would inhibit endothelial cell migration (see Figure 2), cause endothelial cell G, arrest (see Figure 2) and induce endothelial cell apoptosis (see Figures 2 and 3a), thus resulting in a balance of tumor cell proliferation and apoptosis (see Figures 1a and 3b) during the initial 36 days. This endothelial cell by-pass of tumor resistance was dramatic in the p53-/- mice. Although the DNA of p53-null endothelial cells could have been damaged by the first dose of cyclophosphamide, these endothelial cells would not be expected to arrest cell cycle and did not undergo apoptosis. Thus, new microvessels were formed that allowed tumor growth. The arrest of tumor growth was interpreted to demonstrate that after administration of the second dose of cyclophosphamide in the p53-/- mice, the cumulative DNA damage was sufficient to induce nearly synchronous, p53-independent apoptosis of endothelial cells over most of the tumor bed. The magnitude (partial vs. complete) and timing (3.5 days vs. 3 hours) of tumor cell apoptosis in p53 +/+ vs. p53 -/- mice respectively, appeared to depend upon the ability of endothelial cells in p53 +/+ mice to arrest their cell cycle due to cyclophosphamide-mediated DNA damage and either repair or undergo apoptosis. In contrast, DNA damage would have been replicated before repair in p53 -/- endothelial cells following their first exposure to cyclophosphamide. The second and third exposures of p53 -/- endothelial cells to cyclophosphamide then elicited a near total involution of the tumor vasculature and led to the eradication of large, drug-resistant tumors.

Example 7

Eradication of drug-resistant Lewis lung carcinoma by adding TNP-470 to the antiangiogenic schedule of cyclophosphamide

In order to eradicate drug-resistant tumors, it was necessary to potentiate the anti-endothelial activity of cyclophosphamide by adding TNP-470. TNP-470 (a gift from TAP Holdings Inc., Deerfield, IL) was obtained as a lyophilized

powder of 100 mg drug and 726 mg G2-beta-cyclodextrin. 10.3 mg of lyophilized powder was reconstituted with 0.95 ml sterile 5% glucose in water just prior to administration. The dose of TNP-470 was lowered to 1/7th of the dose used in the prior art, because of the occurrence of severe weight loss when TNP-470 was combined with the antiangiogenic schedule of cyclophosphamide.

This lower dose of TNP-470, 12.5 mg/kg every 6 days, was administered on the same day, or day 1, 2, or 4 following administration of cyclophosphamide (170 mg/kg). All drugs were administered subcutaneously, and TNP-470 was injected subcutaneously at 0.01 ml/g body weight on the opposite flank 30 minutes after cyclophosphamide. The combination of cyclophosphamide and TNP-470 on the same day of the six-day cycle proved most efficacious (data not shown). Following 7 cycles of cyclophosphamide on the antiangiogenic schedule with TNP-470, approximately 70% of treated mice received daily subcutaneous administered of dextrose/saline (10% glucose in 75 mM NaCl) for 3-5 days, because during this brief time period the mice ate and drank poorly.

After seven cycles of the combination antiangiogenic therapy described above, in five experiments, drug-resistant Lewis lung carcinoma was eradicated in 32/38 (84%) of mice (Figure 1a). All mice had complete regression of drug-resistant Lewis lung carcinoma and only 3/38 mice (8%) developed recurrent primary tumors 14-18 days after completion of therapy. Another 3/38 mice (8%) died of toxicity within ten days of the completion of therapy. Although these mice died due to severe ataxia and had no evidence of tumor recurrence, these ataxic mice were unable to be evaluated for tumor recurrence and were considered treatment failures.

No tumor relapses occurred beyond 18 days after therapy was completed. However, sterilization of cages, food, and water by autoclaving was found to be critical. In two experiments performed without autoclaving, tumor eradication

occurred in 20/20 mice, yet only 6/20 mice survived a normal life span after completing therapy. The 14/20 mice that succumbed developed pulmonary inflammation 50 ± 6 days after therapy was completed on day 36. Because no tumors recurred beyond 18 days following the completion of therapy, and these mice had no evidence of primary or metastatic tumor at the time of death, the drug-resistant tumors in these 14/20 mice were considered eradicated. It was assumed that the late deaths were in part due to pulmonary endothelial cell damage and immunosuppression caused by cyclophosphamide and complicated by an acquired infection.

Table 1 depicts the long-term survival of 5/6 mice treated with the antiangiogenic schedule of cyclophosphamide and TNP-470. The arrow and note on the graph reflect the recurrence of 1/6 drug-resistant tumor 18 days off therapy. The results of five separate experiments with the antiangiogenic schedule of cyclophosphamide and TNP-470 are detailed in Table 1. Days refer to days following the discontinuance of therapy on day 36 and the # symbol refers to experiments wherein cages, food and water were not sterilized by autoclaving.

	Tumor Recurrence	Toxic Deaths	Late Deaths	Long-term Survivors
n	< 18days	< 10 days	< 36 days	< 100 days
6	0	2	0	4 (549 days)
#6	1	0	0	5 (488 days)
#14	0	0	11	3 (354 days)
6	0	0	3	3 (238 days)
6	2	1	0	3 (102 days)

Example 8

Antiangiogenic Scheduling of 5-fluorouracil and 6-mercaptopurine

The mouse corneal angiogenesis assay was used to screen for antiangiogenic schedules of other chemotherapeutic

agents. 5-fluorouracil (Roche Laboratories, Nutley, NJ) or 6-mercaptopurine ribose phosphate (Sigma, St. Louis, MO) were administered as daily bolus injections of 50 mg/kg/day x 5 days (conventional schedule) or as 50 mg/kg/day continuous infusions (antiangiogenic schedule) via Alzet osmotic minipumps (#2001, Alza Pharmaceuticals, Palo Alto, CA), n=4/group. Pumps were surgically implanted in the peritoneal cavity of large (30-35g) C57B16/J mice on the day prior to corneal pellet implantation. Conventional schedules of both antimetabolites yielded negligible (5 ± 5%) inhibition of angiogenesis. However, the same dose of these antimetabolites as a continuous infusion (antiangiogenic schedule) yielded 66 ± 5% and 60 ± 3% inhibition of angiogenesis for 5-fluorouracil and 6-mercaptopurine ribose phosphate respectively (data not shown).

Example 9

Antiangiogenic Scheduling of doxorubicin and doxil

Doxorubicin hydrochloride (Gensia Laboratories, Irvine CA) or the pegylated liposomal formulation (Doxil, Sequus Pharmaceuticals, Menlo Park, CA) were administered in 5% dextrose in water at 2.5 mg/kg (doxorubicin equivalent dose) once by tail vein injection in SCID mice (Massachusetts General Hospital, Boston, MA) 24 hours after pellet implant, n=6/group. Doxorubicin gave 31 ± 4% and Doxil 42 ± 3%inhibition of angiogenesis (data not shown).

Example 10

Low dose administration of endostatin protein

Endostatin protein is a potent endogenous inhibitor of angiogenesis and tumor growth. Dose response analysis of the efficacy of recombinant human endostatin protein in the early stage of B16BL6 experimental metastasis model revealed significant inhibition of tumor progression at doses as low as 0.05 mg/kg/day. This low dose administration was 2000 fold less than the dose of an administration of 100 mg/kg/day reported in

the prior art to treat primary human xenografts and subcutaneous murine tumors. When assessed in the late stage B16BL6 experimental metastasis model, representing a greater initial tumor burden, tumor progression was inhibited with the administration of endostatin protein at 5 mg/kg/day. In a syngeneic primary tumor model, treatment of mice bearing an orthotopically implanted DA-3 mammary carcinoma with endostatin protein at 5mg/kg/day, resulted in 80% inhibition of tumor growth and development. In addition, subcutaneous injection of the same low dose (5 mg/kg/day) of endostatin protein in mice impregnated subcutaneously with a matrigel plug containing bFGF resulted in a significant decrease in bFGF-induced angiogenesis (approximately 60%). Taken together, these results demonstrate that endostatin protein can exert potent anti-tumor and anti-angiogenic effects at doses far below those reported in the prior art.

Modifications and variations of the present method will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

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Claims

We Claim:

1. A method of treating an angiogenesis-related disease in an individual, comprising administering an effective amount of one or more therapeutic agents to the individual on an antiangiogenic schedule.

2. The method of claim 1, wherein the one or more therapeutic agents are selected from the group consisting of chemotherapeutic agents and antiangiogenic agents.

3. The method of claim 2, wherein the antiangiogenic agents are selected from the group consisting of TNP-470, endostatin protein and angiostatin protein.

4. The method of claim 2, wherein the chemotherapeutic agents are selected from the group consisting of cyclophosphamide, 5-fluorouracil and 6-mercaptopurine.

5. The method of claim 2, wherein the antiangiogenic agent is TNP-470 and the chemotherapeutic agent is cyclophosphamide.

6. The method of claim 1, wherein the disease is a metastatic or angiogenesis-dependent disease.

7. The method of claim 1, wherein the disease is cancer.

8. The method of claim 1, wherein the disease is a tumor, and the administration of the therapeutic agents on the antiangiogenic schedule reduces vascularization or re-vascularization of the tumor.

9. The method of claim 8, wherein the development of drug-resistant tumors is reduced.

10. The method of claim 1, wherein the disease is a tumor, and the administration of the therapeutic agents on the antiangiogenic schedule provides a sustained apoptosis of vascular endothelial cells in the tumor.

11. The method of claim 1, wherein the therapeutic agents are administered every four to eight days until treatment ends.

12. The method of claim 1, wherein the therapeutic agents are administered every six days until treatment ends.

13. The method of claim 1, wherein the therapeutic agents are administered by continuous infusion.

14. The method of claim 1, wherein the therapeutic agents are encapsulated or conjugated to a peptide.

15. The method of claim 1, wherein the therapeutic agents are administered at a low dose of 20 mg/kilogram per day or less.

16. The method of claim 1, wherein the therapeutic agents are administered at a low dose of 0.3 mg/kilogram per day or less.

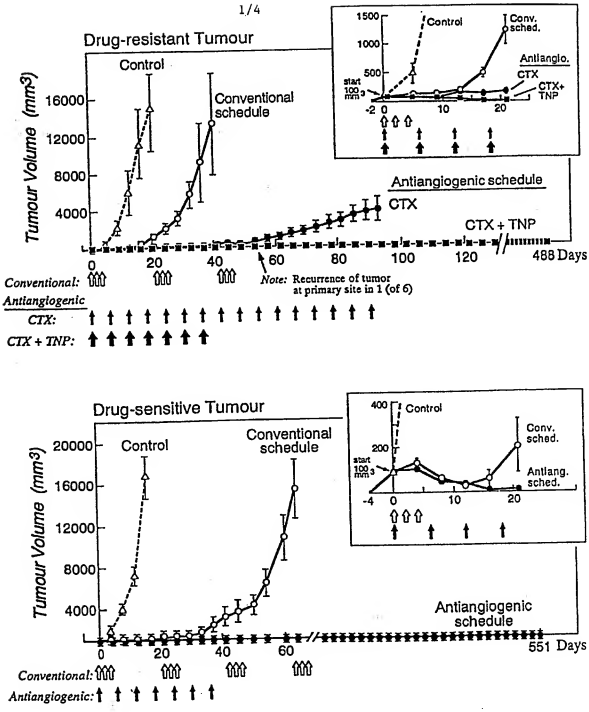
Drug-resistant Tumour
Control
Conventional schedule
Tumour Volume (mm3)
16000
12000
8000
4000
0
20
40
60
80
100
120
488 Days
Antiangiogenic schedule
CTX
CTX + TNP
Conventional:
Note: Recurrence of tumor at primary site in 1 (of 6)
Antiangiogenic
CTX:
CTX + TNP:
1500
1000
500
start 100 mm3
-2
0
10
20
Control
Conv. sched.
Antiangio.
CTX
CTX+ TNP
Drug-sensitive Tumour
Control
Conventional schedule
Tumour Volume (mm3)
20000
16000
12000
8000
4000
0
20
40
60
551 Days
Antiangiogenic schedule
Conventional:
Antiangiogenic:
400
200
start 100 mm3
-4
0
10
20
Control
Conv. sched.
Antiang. sched.

Figure 1

	bFGF	Conc.	Relative migration	Relative cell number	% Apoptosis	Cell cycle (%) G_1	S	G_2-M
Control	—		0	10510 ± 149	1.06	81.71	13.76	4.59
	+		791 ± 34	13005 ± 212	0.51	20.53	74.16	5.32
4HC	+	0.1 μg/ml	437 ± 21	9255 ± 326	0.56	11.03	87.02	1.95
	+	1.0 μg/ml	182 ± 12	6780 ± 208	0.65	46.17	53.83	0
	+	10 μg/ml	ND	2050 ± 159	19.92	78.99	13.77	7.24

Figure 2

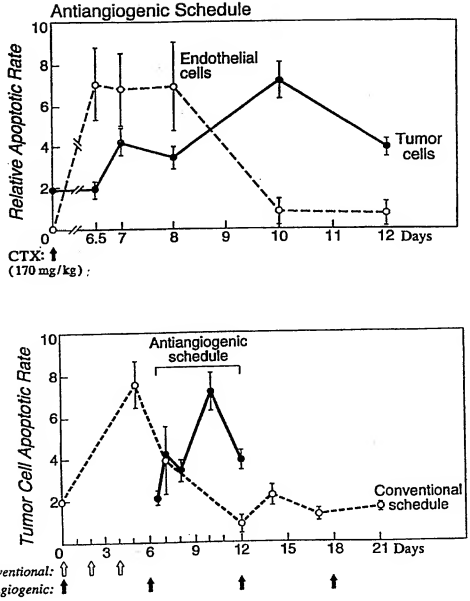
Antiangiogenic Schedule
Relative Apoptotic Rate
Endothelial cells
Tumor cells
Days
CTX:
(170 mg/kg)
Tumor Cell Apoptotic Rate
Antiangiogenic schedule
Conventional schedule
Conventional:
Antiangiogenic:

Figure 3

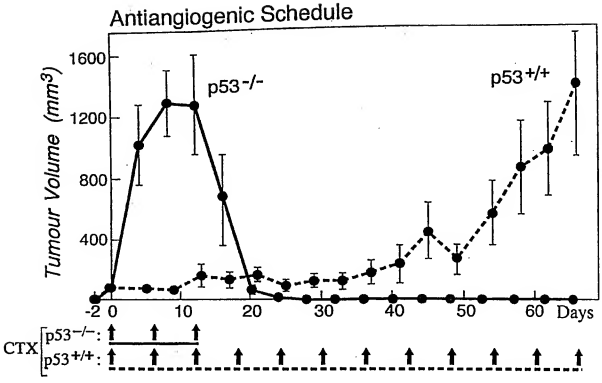
Antiangiogenic Schedule
Tumour Volume (mm3)
1600
1200
800
400
p53-/-
p53+/+
-2 0 10 20 30 40 50 60 Days
CTX
p53−/−:
p53+/+:

Figure 4

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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12 September 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/034174 A3

(54) Title: METHODS FOR ADMINISTRATION OF THERAPEUTIC AGENTS ON AN ANTIANGIOGENIC SCHEDULE

(57) Abstract: The present invention provides compositions and methods for the treatment of diseases associated with the abnormal growth of cells, and more specifically cancer. The compositions and methods of the present invention provide a decreased dosage of chemotherapeutic and antiangionic agents. In particular, the present invention provides methods of administering low doses of endostatin protein. Also included are methods of antiangiogenic scheduling of one or more therapeutic agents.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/30742

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/39 A61K31/675 A61K31/505 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, CANCERLIT, SCISEARCH, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	BROWDER TIMOTHY ET AL: "Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer." CANCER RESEARCH, vol. 60, no. 7, 1 April 2000 (2000-04-01), pages 1878-1886, XP001026181 ISSN: 0008-5472 the whole document ---	1-16
X	US 4 842 855 A (YOUNGNER JULIUS S ET AL) 27 June 1989 (1989-06-27) column 7, line 57 - line 60 ---	1,2,4, 6-11
X	WO 99 13053 A (UNIV YALE ;VION PHARMACEUTICALS INC (US)) 18 March 1999 (1999-03-18) page 43, line 10 - line 11 ---	1,2,4, 6-11

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
O document referring to an oral disclosure, use, exhibition or other means
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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2001	16/01/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Escolar Blasco, P

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	PLACIDI LAURENT ET AL: "Metabolic drug interactions between angiogenic inhibitor, TNP-470 and anticancer agents in primary cultured hepatocytes and microsomes." DRUG METABOLISM AND DISPOSITION, vol. 27, no. 5, May 1999 (1999-05), pages 623-626, XP002186178 ISSN: 0090-9556 Introduction -second paragraph Conclusion ---	1-11
X	TEICHER B A ET AL: "COMPARISON OF SEVERAL ANTIANGIOGENIC REGIMENS ALONE AND WITH CYTOTOXIC THERAPIES IN THE LEWIS LUNG CARCINOMA" CANCER CHEMOTHERAPY AND PHARMACOLOGY, SPRINGER VERLAG, BERLIN, DE, vol. 38, no. 2, 1996, pages 169-177, XP000973352 ISSN: 0344-5704 abstract ---	1-11
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1984 PETERS W P ET AL: "PHASE I TRIAL OF COMBINATION THERAPY WITH CONTINUOUS-INFUSION 6 METHYL MERCAPTOPURINE RIBOSIDE AND CONTINUOUS-INFUSION 5 FLUOROURACIL" Database accession no. PREV198579042131 XP002186179 abstract & CANCER CHEMOTHERAPY AND PHARMACOLOGY, vol. 13, no. 2, 1984, pages 136-138, ISSN: 0344-5704 ---	1,2,4, 6-10,13
X	RANSON M ET AL: "Phase II dose-finding trial of CAELYX(StealthR) liposomal doxorubicin HCL) in the treatment of advanced breast cancer" EUROPEAN JOURNAL OF CANCER, PERGAMON PRESS, OXFORD, GB, vol. 33, September 1997 (1997-09), page S148 XP004282899 ISSN: 0959-8049 abstract --- -/--	1,2, 6-10, 13-15

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	VAAGE J ET AL: "TISSUE DISTRIBUTION AND THERAPEUTIC EFFECT OF INTRAVENOUS FREE OR ENCAPSULATED LIPOSOMAL DOXORUBICIN ON HUMAN PROSTATE CARCINOMA XENOGRAFTS" CANCER, AMERICAN CANCER SOCIETY, PHILADELPHIA, PA, US, vol. 73, no. 5, 1994, pages 1478-1484, XP000979547 ISSN: 0008-543X Treatment schedule abstract; table 1 page 1483, right-hand column ---	1,2, 6-11,14, 15
X	O'REILLY M S ET AL: "ENDOSTATIN: AN ENDOGENOUS INHIBITOR OF ANGIOGENESIS AND TUMOR GROWTH" CELL, CELL PRESS, CAMBRIDGE, NA, US, vol. 88, no. 2, 24 January 1997 (1997-01-24), pages 277-285, XP000652213 ISSN: 0092-8674 abstract page 279, right-hand column, paragraph 2 ---	1-3, 6-10,15, 16
X	TOMASZEWSKI J E ET AL: "PHARMACOKINETICS AND RANGE-FINDING TOXICITY STUDIES OF RECOMBINANT HUMAN ENDOSTATIN TM IN CYNOMOLGUS MONKEYS" PROCEEDINGS OF THE ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, NEW YORK, NY, US, vol. 40, March 1999 (1999-03), pages 385-386, XP000982526 ISSN: 0197-016X abstract -----	1-3, 6-10,13, 15

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 1-16 are directed to a method of treatment of the human or animal body, the search has been carried out and based on the alleged effects of the compounds.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

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Continuation of Box I.2

Present claims 1,2 and 6-16 relate to an extremely large number of possible therapeutic agents and administration patterns. In fact, the claims contain so many options that a lack of clarity within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claims impossible. Consequently, the search has been carried out for those parts of the application which do appear to be clear, namely the examples.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/30742

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4842855 A	27-06-1989	NONE	
WO 9913053 A	18-03-1999	US 6080849 A	27-06-2000
		AU 9380798 A	29-03-1999
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		ZA 9808289 A	22-03-1999